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Original article

Preserved bone mass in ovariectomized rats treated with parathyroid-hormone-related peptide (1-34) and (107-111) fragments

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Summary — The effect of synthetic human PTHrP (1-34) or (107-111) fragments on bone loss was studied in rats, one month after ovariectomy (OVX). Four groups of 7–8-month-old rats were treated sc daily for 13 d with PTHrP (1-34) or (107-111) at the dose of 1 or 3 nmol/100 g body weight. Sham-operated (SHO) and control OVX rats received solvent alone. In our conditions, at the lowest dose, neither (1-34) nor (107-111) fragments had any significant effect. However, at the dose of 3 nmol/100 g daily for 13 d both treatments significantly increased femoral dry weight, ash weight, Ca content and densitometry of the femur. The effect of PTHrP (1-34) mainly resulted from increased cortical and trabecular bone (% recovery: 98.25 and 105.23%, respectively). For the PTHrP (107-111) fragment, a positive effect was only demonstrated on the cortical bone (98.25% recovery). The results of this study demonstrate that both hPTHrP (1-34) and (107-111, osteostatin) fragments are positive for bone when administered at the dose of 3 nmol/100 g body weight/d for 13 d to adult OVX rats.

PTHrP / ovariectomy / rat / bone

Résumé — Les fragments (1-34) et (107-111) du peptide apparenté à l'hormone parathyroïdienne préservent la masse osseuse de la rate ovariectomisée. *Les effets des fragments (1-34) et (107-111) du PTHrP synthétique humain sur la perte osseuse induite par ovariectomie ont été étudiés chez la rate. Quatre groupes de rates âgées de 7 à 8 mois ont reçu un mois après ovariectomie par voie sc pendant 13 j les fragments (1-34) et (107-111) de PTHrP aux doses de 1 ou 3 nanomoles/100g/j de poids vif. La dose la plus faible n'a eu aucun effet significatif par rapport aux animaux ovariectomisés recevant le solvant. Administrés à la dose de 3 nanomoles/100 g/j pendant 13 j, les 2 peptides augmentaient le poids sec, la teneur en calcium et la densité du fémur. Les effets du fragment (1-34) provenaient d'une augmentation de l'os cortical et trabéculaire dont les pourcentages de récupération étaient respectivement de 98,25% et 105,23%. Le fragment (107-111) n'avait un effet qu'au niveau de l'os cortical.*

PTHrP / ovariectomie / rate / os

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INTRODUCTION

The action of parathyroid hormone (PTH) on the skeleton is paradoxical because it directly and indirectly affects bone formation and resorption (Parsons, 1976). The first evidence that bone resorption is a direct response to PTH was obtained by Barnicot half a century ago (Barnicot, 1948). Several studies have confirmed this result (Parsons, 1976). However, when PTH is intermittently administered at low doses, it is anabolic for bone in normal and osteoporotic rats (Burrows, 1938; Hock and Wood, 1991; Kimmel *et al,* 1993), in adult dogs (Parsons and Reit, 1974) and in osteoporotic humans (Reeve *et al,* 1980).

The discovery of PTH-related peptide (PTHrP) was the result of investigations into the mechanisms by which some cancers in humans (Suva *et al,* 1987; Mangin *et al,* 1988) or animals (Rosol *et al,* 1983) cause hypercalcemia without necessarily metastasizing to bone. PTHrP has similar effects to PTH on bone cells (Stewart *et al,* 1988; Thompson *et al,* 1988; Civitelli *et al,* 1989). Although some studies indicate that the synthetic human PTHrP (hPTHrP) (1-34) fragment might be less potent that hPTH (1-34) as an anabolic agent for bone *in vivo* (Fukayama *et al,* 1988; Hock *et al,* 1989), hPTHrP (1-74) increases bone mass when injected at the dose of 1 or 2 nmol/100 g body weight/d for 12 d in young growing male rats (Weir *et al,* 1992). From studies of the action of the hPTHrP (1-141) molecule on populations of osteoclasts disaggregated from neonatal rat bones, it has been shown that hPTHrP (1-141) had a direct effect on osteoclasts in inhibiting bone resorption, whereas no such inhibition was obtained with the synthetic amino-terminal peptides hPTHrP (1-34) and (1-108). This osteoclast inhibitory activity was localized to a carboxyl-terminal fragment: hPTHrP (107-139) (Fenton *et al,* 1991a). The name osteostatin has been proposed for the highly conserved pentapeptide, TRSAW, corresponding to residues (107-111) in hPTHrP (107-139) responsible for this inhibition, which was assessed by quantification of resorption pits formed by osteoclasts disaggregated from neonatal rat bones and settled onto thin slices of devitalised bovine cortical bone (Fenton *et al,* 1991b). However, another recent work reports that osteostatin does not affect basal or PTHrP (1-34)-stimulated ^{45}Ca release from prelabelled neonatal mouse calvariae, while salmon calcitonin was a potent inhibitor of both basal and stimulated ^{45}Ca release from bone (Sone *et al,* 1992). Thus, in the present work, we have compared the effects of synthetic hPTHrP (1-34) and osteostatin on bone loss induced by ovariectomy in adult rats (Kalu, 1991).

MATERIALS AND METHODS

Experimental design

Forty-two retired breeder female Wistar rats, aged 7–8 months, weighing 386 ± 10 g were used. On day 0, 35 animals were ovariectomized (OVX) by the dorsal approach under chloral hydrate anaesthesia. The 7 remaining rats were sham-operated (SHO). On day 30, the 35 OVX rats were randomly divided into 5 groups of 7 animals. From day 30 to 42, they were injected once daily sc either with 1 nmol/100 g body weight PTHrP (1-34), or with 3 nmol/100 g body weight PTHrP (1-34) (*ie* 40 and 120 µg/kg body weight, respectively), or with 1 nmol/100 g body weight PTHrP (107-111), or with 3 nmol/100 g body weight PTHrP (107–111) (*ie* 6.2 and 18.6 µg/kg body weight, respectively). During that time, the 14 other rats (7 OVX + 7 SHO) received in the same way, the solvent alone (Nacl 0.9% with 0.01% bovine serum albumin; 0.1 ml/100 g body weight). Rats were weighed every 3 d, and the dose administered to each animal was adjusted to maintain the correct dosage. Both fragments from synthetic hPTHrP were purchased from Saxon Biochemicals (Hannover, Germany).

Each rat was individually housed in a metabolic cage (IFFA Credo, l'Arbresle, France) and fed with UAR Elevage Chow (9 g calcium

(Ca) and 7.8 g inorganic phosphorus (P)/kg diet). To avoid any influence of OVX on food intake, only 30 g of food was given daily to each rat during the experimental period. This was the mean daily food consumption by these animals during the week before OVX. During a balance study from days 36 to 42 the urinary excretion of Ca and P was measured for each rat. On day 43, the rats were killed by exsanguination (cardiac puncture) under chloral anaesthesia. After centrifugation, the plasma was frozen until analysis. The uterine horns of each animal were collected and immediately weighed. Both legs were disarticulated at the hip. Femurs and tibias were collected and cleaned of soft tissue. The right bones were placed in 70% ethanol for histomorphometry. The left femur was defatted in acetone for 7 d, dried and weighed. After ashing at 550°C for 12 h, the ashes were dissolved in 2 M HCl for Ca measurement.

The Ca concentrations in bone, urine, feces and blood plasma were measured by atomic absorption spectrophotometry (Perkin Elmer 400). Phosphorus in urine and plasma was measured using a spectrocolorimeter (Secoman, Domont, France).

Analysis

The femoral bone density was measured at 19 uniform intervals (1/8 inch) from the distal to the proximal end using a Norland single photon absorptiometer and expressed per bone width for these sites.

After total dehydration the right tibia and the right femur were embedded in methyl methacrylate (Miller *et al,* 1989). In order to characterize static cancellous bone, frontal sections of the distal femur were cut with a saw and stained with Von Kossa's reagent and analysed with an automated television microscope image analysis system, as previously described (Miller and Jee, 1975). Histomorphometry indices (area and width of the cortical bone, cancellous bone area, thickness of trabeculae as well as osteoclastic area and osteoid percentage of the cancellous bone) were determined from the cross-section at the tibio-fibular junction. The sections were quantified using a digitizing tablet and a microcomputer with histomorphometry software (Anderson, 1982).

Results are expressed as the mean ± the standard error mean (SEM). Statistical evaluation of the data was performed by analysis of variance using the Dunnett's *t* test (all values are given at a 95% level of significance). The OVX-treated rats were compared with both SHO and OVX controls.

RESULTS

The OVX rats were food-restricted and so their body weight at death (395 ± 23 g) was no different from that of the SHO animals (412 ± 30 g).

The atrophied uterine horns (322 ± 18 mg ($n = 35$) *vs* 1 107 ± 47 mg ($n = 7$); $p < 0.01$) confirmed successful ovariectomy in operated rats.

In our experimental conditions, no treatment had any significant effect upon either body weight at death or on plasma Ca (10.0 ± 0.5 mg/dl ($n = 42$; 6 groups of 7 animals)) and P concentrations (5.2 ± 0.8 mg/dl ($n = 42$; 6 groups of 7 animals)). The Ca (6.0 ± 1.3 mg/d; $n = 294$) and P urinary excretion (3.6 ± 1.1 mg/d; $n = 294$) were not significantly modified either (mean values for the 42 rats during the 7 d balance study).

Ovariectomy caused a decline in femoral dry weight, ash weight, Ca content and mineral density (table I). These parameters were not different in OVX rats treated with solvent alone and in OVX rats treated with the lowest dose (1 nmol/100 g body weight/d for 13 d) of PTHrP (1-34) or (107-111) (table I). In OVX rats given the highest dose (3 nmol/100 g body weight/d for 13 d) of PTHrP (1-34) or (107-111), the dry weight, ash weight, Ca content (% recovery (defined as Ca content in femoral bone from treated rats related to that measured in SHO controls): 101.09 and 97.28%, respectively) and densitometry (total bone density (g/cm/cm): 0.296 ± 0.017 and 0.279 ± 0.014 for PTHrP (1-34) and (107-111) fragments and % recovery 101.02 and 95.22%, respectively)

Table I. Physicochemical parameters of the femurs.

	Dry weight (mg)	*Ash weight (mg)*	*Ca content (mg)*	*Bone density (g/cm/cm)*
SHO + solvent	744 ± 15	486 ± 23	184 ± 3	0.293 ± 0.004
OVX + solvent	704 ± 9*	459 ± 21*	177 ± 4*	0.269 ± 0.005*
OVX + PTHrP (1-34) (1 nmol/100 g)	718 ± 17*	448 ± 20*	178 ± 2*	0.275 ± 0.007*
OVX + PTHrP (1-34) (3 nmol/100 g)	748 ± 5**	486 ± 69	186 ± 6**	0.296 ± 0.007**
OVX + PTHrP (107-111) (1 nmol/100 g)	681 ± 24*	437 ± 26*	168 ± 9*	0.276 ± 0.005*
OVX + PTHrP (107-111) (3 nmol/100 g)	717 ± 12	478 ± 14	181 ± 3	0.279 ± 0.006*

Means ± SEM; * $p < 0.05$, comparison with SHO rats; ** $p < 0.05$, comparison with the OVX + solvent group; no significant difference could be demonstrated between OVX + PTHrP (1-34) and OVX + PTHrP (107-111) groups; the right femur was used for bone density measurement, and the left for the other parameters.

of the right femur increased up to values measured in control SHO rats (0.293 ± 0.09) (table I), *ie* a 105 and 101% increase compared with OVX control rats (0.269 ± 0.01), respectively. The effects induced by both treatments on the measured parameters were never significantly different (table I). The effects of the PTHrP (1-34) fragment on bone densitometry mainly appeared at both ends of the femur (fig 1).

Ovariectomy significantly decreased down the cortical bone area, compared with sham surgery (table II). The PTHrP (1-34) and (107-111) fragments, given at the dose of 1 nmol/100 g body weight/d, did not have any significant effect upon cortical bone parameters, which were not different from those measured in OVX rats given solvent alone. However, at the dose of 3 nmol/100 g body weight/d, both PTHrP (1-34) and (107-111) similarly increased cortical bone area in OVX rats, bringing it back to those measured in SHO animals (% recovery 98.25%; table II); *ie* a 109% increase compared with OVX control rats. Nevertheless, no significant change concerning the cortical width was observed (table II).

The trabecular bone of the femur was decreased by OVX. The epiphyseal and the metaphyseal bone volume were significantly

Table II. Morphometric parameters measured at the mid-diaphyseal shaft of the right tibia.

	Cortical area (μ^2)	*Cortical width (μ)*
SHO + solvent	4.56 ± 0.09	0.627 ± 0.011
OVX + solvent	4.12 ± 0.12*	0.626 ± 0.026
OVX + PTHrP (1-34) [a]	4.48 ± 0.11**	0.619 ± 0.019
OVX + PTHrP (107-111) [a]	4.48 ± 0.10**	0.639 ± 0.029

[a] 3 nmol/100 g body weight/d; means ± SEM; * $p < 0.05$, comparison with SHO rats; ** $p < 0.05$, comparison with the OVX + solvent group, no significant difference could be demonstrated between OVX + PTHrP (1-34) and OVX + PTHrP (107-111) groups.

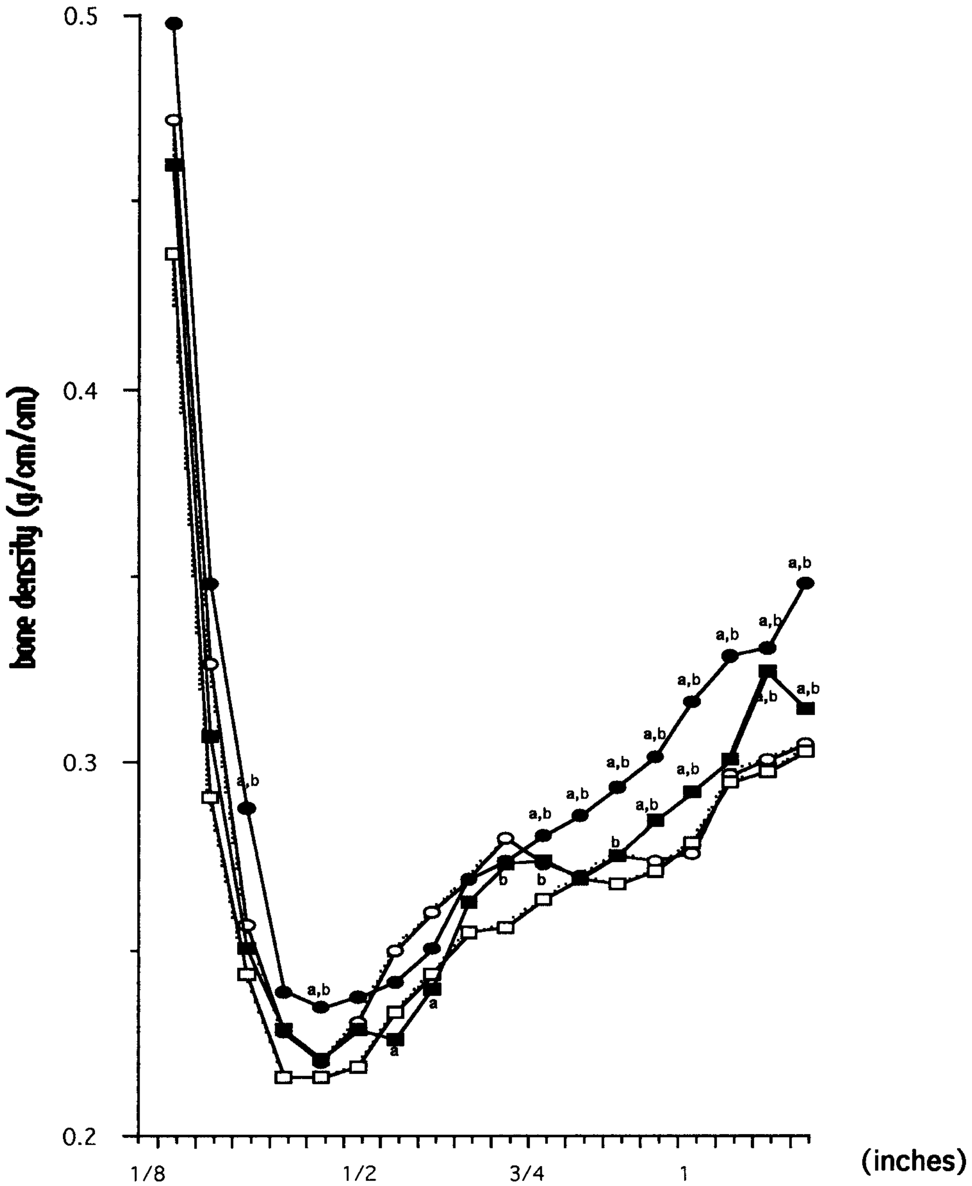

Fig 1. Bone mineral content per bone width (g/cm/cm) measured by single photon absorptiometry at intervals distal to the proximal femur of OVX (open squares), OVX + PTHrP (107-111) (3 nmol/100 g body weight/d; solid squares) and OVX + PTHrP (1-34) (3 nmol/100 g body weight/day; black circles) rats (each point represents the mean value for the 7 rats of each group). For clarity SEM are not shown; [a,b] $p < 0.05$, comparison with SHO rats (open circles) or OVX rats.

reduced compared with SHO animals. Actually this could be explained by a higher turnover, the percentage of osteoid and the number of osteoclasts per area being both significantly increased (table III; fig 2). While the lowest dose of PTHrP (1-34) or (107-111) did not have any significant effect on trabecular bone, the administration of PTHrP (1-34) at the dose of 3 nmol/100 g body weight/d increased bone volume up to the level of SHO animals, the trabeculae being even thicker than in the OVX group (table III; fig 2). On the contrary, PTHrP (107-111) at 3 nmol/100 g body weight/d for 13 d did not improve the bone volume and compared to OVX animals, trabeculae were even thinner. This treatment was associated with a lower percentage of osteoid and more numerous osteoclasts than with PTHrP (1-34) given at the same dose (table III; fig 2).

DISCUSSION

In our food-restricted rats, OVX did not induce a significant increase in body weight during the 43 d following surgery. However, similar skeletal changes have been demonstrated in OVX rats whether pair-fed, weight-matched or fed *ad libitum* and likewise pair-feeding has no qualitative effects on skeletal changes after OVX (Arjmandi *et al*, 1993). The full length recombinant PTHrP molecule and its (1-34) amino-terminal fragment share the same effects on cytosolic Ca ion mobilization and adenylate cyclase activation in rat osteoblast-like cells (Donahue *et al*, 1989). As shown by the daily Ca intake of each rat (about 270 mg), the 30 g of food (9 mg Ca/g diet) given daily to each animal were totally consumed. However, since our rats were not pair-fed, it is possible that subtle changes in urinary Ca and P excretion were not detected in the present study.

As previously reported (Kalu, 1991; Miller *et al*, 1991; Kimmel *et al*, 1993), OVX induced osteopenia in rats (tables I–III; fig 2). Femoral dry weight, ash weight, Ca content and bone mass were significantly decreased in OVX rats. However, while both PTHrP (1-34) and (107-111) were inefficient at the dose of 1 nmol/100 g body weight/d for 13 d, at the highest dose, they brought these parameters back to values measured in SHO rats (table II). In a 4-week-old intact male Sprague-Dawley rats, the hPTHrP (1-74) fragment given sc at the doses of 1 or 2 nmol/100 g body weight/d for 12 d caused a slight but significant increase in femoral bone weight related to an increase in femoral Ca and hydroxyproline content (Weir *et al*, 1992). Our results demonstrate that hPTHrP (1-34) and (107-111) similarly pre-

Table III. Morphometric parameters of the cancellous bone measured at the distal epiphysis of the femur after erasing cartilage tissue (* Parfitt *et al*, 1987).

	Bone surface (%)	*Trabecular thickness (perimeter/area)*	*Osteoclast surface (number of cells/mm²)*	*Osteoid surface (%)* *
SHO + solvent	6.2 ± 0.5	19.51 ± 1.15	0.61 ± 0.30	7.3 ± 4.8
OVX + solvent	5.6 ± 0.5[a]	16.41 ± 1.50[a]	1.11 ± 0.23[a]	20.0 ± 3.5[a]
OVX + PTHrP (1-34) [x]	6.8 ± 1.2[c]	15.77 ± 1.37[ac]	0.56 ± 0.19[bc]	40.4 ± 10.9[abc]
OVX + PTHrP (107-111) [x]	4.9 ± 0.6[ab]	20.69 ± 2.42[b]	1.62 ± 0.67[a]	2.4 ± 0.6[b]

Means ± SEM; [a] $p < 0.05$, comparison with SHO rats; [b] $p < 0.05$, comparison with the OVX + solvent group; [c] $p < 0.005$, comparison with PTHrP (107-111) group. [x] 3 nmol/100 g body weight/d, for 13 d.

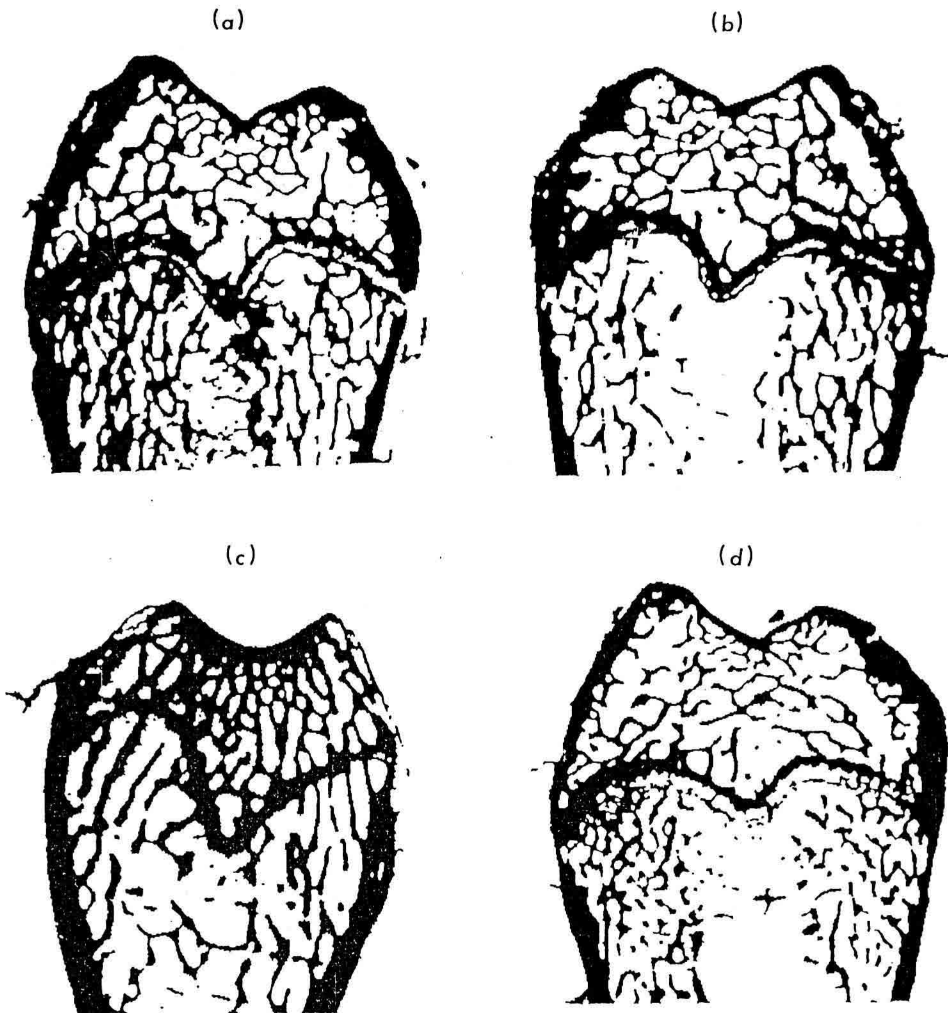

Fig 2. Representative pictures obtained by image analysis (Miller and Jee, 1975) from frontal sections through the distal femur stained with Von Kossa's reagent from SHO (a), OVX (b), OVX + PTHrP (1-34) (3 nmol/100 g body weight/d (c) and OVX + PTHrP (107-111) (3 nmol/100 g body weight/d (d) rats.

vent bone loss *in vivo* when administered at the dose of 3 nmol/100 g body weight/d for 13 d to OVX rats. When injected at the dose of 2 nmol/100 g/d for 12 d in young male Sprague-Dawley rats PTHrP did not increase bone mass or bone-forming surfaces. However, at a 4 fold higher dose it increased trabecular bone mass by 24–36% (Hock *et al,* 1989). In our experiment, the (1-34) fragment acted both at the trabecular (table III; fig 2) and the cortical levels as shown by the cortical surface at the tibiofibular junction (table II). Murrills *et al* (1990) examined the effect of PTHrP (1-40) frag-

ment on osteoclastic resorption of bone slices *in vitro* and demonstrated that it caused a decrease in size of osteoclast resorption lacunae compared with control, which might also play a role in the positive effect of the hormone. In the same way, in our animals, at the epiphysis level, while the osteoid area was increased by the (1-34) treatment (3 nmol/100 g body weight), the number of osteoclasts per area was lower than in OVX controls (table III).

Although both fragments act in the same way at the cortical level to increase bone mass (table II), in contrast, the PTHrP (107-111) fragment decreased bone formation at the trabecular level (based on measures of osteoid surface) and increased bone resorption (based on osteoclastic surfaces) (table III). This might explain why both bone area and trabecular thickness (table III, fig 2) were also decreased by this treatment. However, in our experimental conditions, the PTHrP (107-111) fragment given daily for 13 d at the dose of 3 nmol/100 g body weight, increased femoral Ca content and bone density (table I), bringing the cortical bone area back to that measured in SHO controls (table II), in accordance with the results from Fenton *et al* (1991a, b). This might partly explain why Sone *et al* (1992) found no effect in an attempt to inhibit murine calvarial resorption by osteostatin.

In conclusion, our results demonstrate that PTHrP (1-34) treatment (3 nmol/100 g body weight, daily for 13 d) increased bone mass in adult OVX rats. The global effect of the same amount of the (107-111) fragment also seems to be anabolic, as shown by the increase in femoral bone mass and Ca content. Thus, in our conditions, the anabolic effect of osteostatin on cortical bone probably exceeded its resorbing action at the trabecular level. However, further studies are required to determine if PTHrP prevents bone loss or has an anabolic effect by increasing bone formation.

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